

Emmi Tiilikainen

Extracellular matrix stiffness in regulation of intestinal stem cell function in 3D culture

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<p>Stem cells have the significant ability to divide interminably and to generate all the specialized cell types in the body. Characteristics, such as being undifferentiated and having the ability to self-renew and to generate daughter cells with distinct cell fates, separate stem cells from other cells types.</p> <p>Adult stem cells maintain tissue-renewal. The intestinal epithelium undergoes renewal in five days, hence providing a suitable basis to study intestinal stem cells <i>in vitro</i>. Intestinal stem cells, locating in the bottom of intestinal crypts, generate daughter cells that differentiate while migrating towards the top of the villus. When cultured in three-dimensional matrix, intestinal stem cells grow into 3D intestinal organoids that mimic original <i>in vivo</i> intestinal stem cell niche conditions.</p> <p>Multiple studies have demonstrated that extracellular matrix, secreted locally by the cells, affects stem cell function and proliferation by providing mechanical support and by enabling cell-to-cell signaling pathways. The question, how physical properties of extracellular matrix such as stiffness affects precisely on the intestinal stem cell function, is still unanswered.</p> <p>This engineering thesis was carried out in Pekka Katajisto's research group in the Institute of Biotechnology at the University of Helsinki. The aim of this thesis was to study how the stiffness of extracellular matrix regulates the stem cell function when culturing in three-dimensional matrix.</p> <p>Murine intestinal stem cells were isolated and cultured in Matrigel-based 3D culture that mimics <i>in vivo</i> extracellular matrix conditions. The stiffness of the culture matrix was modified by varying the Matrigel concentration and by cross-linking the matrix with stiffness-increasing calcium alginate.</p> <p>The results indicate that the stiffness of the Matrigel-based 3D matrix seems to influence on the width of the intestinal crypts and therefore it might have a role in regulation of the intestinal stem cell function. The findings indicate that the influence of matrix stiffness to other parameters was minor and strictly directional. Further experiments are needed in order to adapt these results to <i>in vivo</i> conditions.</p>	
Keywords	stem cell, microenvironment, organoid, extracellular matrix, 3D culture, intestine

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<p>Kantasoluilla on kyky jakautua loputtomasti ja tuottaa elimistön eri tehtäviin erikoistuneita soluja. Erilaistumattomuus, kyky uusiutua rajattomasti ja kyky luoda solunjakautumisessa sekä uusi kantasolu että erilaistuva tytärsolu ovat ominaisuuksia, jotka erottavat kantasolut elimistön muista solutypeistä.</p> <p>Aikuisen kantasoluilla on merkittävä rooli kudosten uusiutumisessa. Ohutsuolen epiteeli uusiutuu täysin viidessä päivässä, ollen kehomme nopeimmin uusiutuva kudos. Ohutsuolen kantasolut sijaitsevat Lieberkühnin kryptissa. Kryptan kantasolut tuottavat uusia soluja, jotka erilaistuvat ja siirtyvät ylöspäin kohti villuksen kärkeä. Erittäin nopean uusiutumiskykynsä ansioista ohutsuolen kantasolut tarjoavat sopivan alustan kantasolujen toiminnan tutkimiseen <i>in vitro</i>. Ohutsuolen kantasoluja voidaan viljellä kolmiulotteisessa matriisissa, jolloin ne muodostavat luonnollista ohutsuolen kantasolulokeroa muistuttavia organoideja.</p> <p>Useat tutkimukset ovat osoittaneet, että solujen ympärilleen tuottama soluväliaine vaikuttaa kantasolujen toimintaan tarjoamalla mekaanista tukea sekä mahdollistamalla solujen välisen viestinnän ja solun ulkopuolisten signaalien kulkeutumisen. Se, miten soluväliaineen fysikaaliset ominaisuudet, kuten jäykkyys, vaikuttavat juuri ohutsuolen kantasolujen toimintaan, on kuitenkin vielä epäselvää.</p> <p>Tämä insinöörityö toteutettiin Biotekniikan Instituutissa Pekka Katajiston tutkimusryhmässä Helsingin Yliopistolla. Työn tarkoituksena oli tutkia, miten soluväliaineen jäykkyys vaikuttaa ohutsuolen kantasolujen toimintaan kasvatettaessa niitä kolmiulotteisessa matriisissa.</p> <p>Hiirten ohutsuolen kantasoluja eristettiin ja kasvatettiin luonnollista soluväliainetta jäljittelevässä Matrigeeli-pohjaisessa 3D-matriisissa. Kasvatusmatriisin jäykkyyttä modifioitiin vaihtelemalla Matrigeelin konsentraatiota kasvatusmatriisissa sekä ristisilloittamalla kasvatusmatriisia jäykkyyttä lisäävällä kalsiumalginaatilla.</p> <p>Tulosten perusteella huomattiin, että käytetyn Matrigeeli-pohjaisen kasvatusmatriisin jäykkyys vaikuttaa kasvavien kryptien leveyteen. Tulokset muista määrittämisistä ovat suuntaa antavia ja useampia toistoja vaadittaisiin, jotta saatuja tuloksia voitaisiin soveltaa <i>in vivo</i> -olosuhteisiin.</p>	
Avainsanat	kantasolu, mikroympäristö, organoidi, soluväliaine, 3D-kasvatus, ohutsuoli

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Abbreviations

BMP	Bone morphogenetic protein
CBC cell	Crypt base columnar cell
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGC	Embryonic germ cell
EGF	Epidermal growth factor
ESC	Embryonic stem cell
iPSC	Induced pluripotent stem cell
ISC	Intestinal stem cell
LGR5	Leucine-rich G protein-coupled receptor 5
PBS	Phosphate-buffered saline
RSPO	R-spondin
RT	Room temperature
TAC	Transit amplifying cell
3D	Three-dimensional

1 Review of the literature

1.1 Stem cells

Stem cells have the remarkable ability to divide and generate the vast array of specialized cell types in the body. Stem cells have a significant role in tissue self-renewal, since the outer layers of several tissues are replaced tens to thousands times during a human lifetime. This kind of internal repair system can be maintained only if some cells of the tissue are self-renewing: daughter cells of a dividing stem cell can either remain as a stem cell or differentiate into organ- or tissue-specific cell with special functions. [Alberts *et al.*, 2002; Stem Cell Basics: Introduction. 2002]

Regardless of their environmental source, there are three defining characteristics that separate stem cells from other cell types. First, stem cells are undifferentiated. Due to their lack of tissue-specific features, stem cells are not able to carry out specialized functions. Second, unspecialized stem cells can divide without limits and renew themselves for long periods by replicating or proliferating. Long-term self-renewal gives stem cells the ability of duplicating themselves to the same non-specialized form during months or even years. [What are the unique properties of all stem cells?, 2009.] Finally, when dividing, each produced daughter cell can either become a terminally differentiated cell by going through several stages or remain unspecialized stem cell. This specialized cells forming process is called differentiation. [What are the unique properties of all stem cells?, 2009; Alberts *et al.*, 2002]

1.1.1 Embryonic stem cells (ESCs)

The development of mammalian embryo begins when a sperm and an egg fuse in the process called fertilization. The fertilization initiates embryogenesis. In order to grow, the fertilized egg, called *zygote*, begins to divide repeatedly forming 2-, 4- and 8-cell stages. The cells produced during these early stages of the embryonic development are called *totipotent* – they can give rise to any cell types in the embryo or in the adult body (including germ cells). [Alberts *et al.*, 2002]

At the 16-cell stage, the cells of the zygote form a solid ball of cells called *morula*. The morula transforms itself onwards into a *blastocyst* (or *blastula*) – a form where an internal cavity becomes visible. After the third cell division, the totipotent cells have given rise to *pluripotent* cells adhered to one side of the blastocyst. This inner clump of cells is called the *inner cell mass* (ICM). [Alberts *et al.*, 2002]

According to the name, embryonic stem cells (ESCs) are cells that have been derived from the blastocyst stage early mammalian embryo. ESCs from the ICM of blastocyst are pluripotent: they have the ability to differentiate into any type of cell of the body, except the cells of placenta. The pluripotency distinguishes ESCs from the adult stem cells found in adult tissues (see chapter 1.1.2).

In the process called gastrulation, the symmetric one-layered blastula folds inward and enlarges to create a *gastrula*. The inner pluripotent cell mass of the blastocyst is able to differentiate into three primary germ layers: ectoderm, endoderm and mesoderm.

1.1.2 Adult stem cells

Adult stem cells, also known as tissue stem cells, are cells that can be identified in several tissues and organs (such as brain, bone marrow, skin, teeth, blood vessels, heart and gut) in the mammalian body. Contrary to the ESCs, the adult stem cells (or somatic stem cells, referring to cells of the body excluding the germ cells, eggs and sperm) are undifferentiated cells that have the ability to self-renewal and to give rise to some or all the cell types of the given tissue. [What are adult stem cells?, 2012; Alberts *et al.*, 2002] The key functions of the adult stem cells are to maintain and repair organs and tissues in which they are found from [What are adult stem cells?, 2012]. By generating new specialized cells, the adult stem cells are compensating the tissue lost in cases like injuries, diseases or during normal continuous tissue regeneration and aging [reviewed in Snippert & Clevers, 2011].

Hence, the adult stem cells are called *multipotent stem cells* being the progeny of pluripotent stem cells and giving rise to various but limited range of cells within a tissue and eventually becoming lineage-committed cells [Alberts *et al.*, 2002]. The multipotent stem cells such as hemopoietic stem cells, which can give rise to several blood cell types, or mesenchymal stem cells, which can differentiate into bone, cartilage and fat cells, produce even more differentiated progeny called *unipotent stem cells*: terminally

differentiated stem cells devoting the capacity to specialize into only one cell type such as epidermal stem cells.

A number of recent studies [Thorel *et al.*, 2010; Kragl *et al.*, 2009] have demonstrated that certain adult stem cells might undergo a process called *transdifferentiation* – a regenerative phenomenon where stem cells from one tissue transform into cells of another [What are adult stem cells?, 2012]. The terminally differentiated cells convert, without the pluripotent stage, into a functionally distinct cell type. This process, also known as lineage reprogramming, has been reported in particular vertebrates [reviewed in Kikuchi, 2015], but the relevance of transdifferentiation for normal physiology is still under investigation.

1.1.3 Induced pluripotent stem cells (iPSCs)

Induced pluripotent stem cells (iPSCs) are embryonic stem cell-like cells genetically reprogrammed from differentiated adult cells [What are induced pluripotent stem cells?, 2009] and they exhibit pluripotency. In addition, the iPSCs are capable of differentiation into the three primary germ cells and they resemble to the ESCs in gene expression and morphology [Park *et al.*, 2008; What are induced pluripotent stem cells?, 2009]. The iPSCs can be routinely generated from mouse embryonic fibroblasts and adult mouse fibroblasts [Takahashi & Yamanaka, 2006] as well as from adult human dermal fibroblasts [Takahashi *et al.*, 2007; Park *et al.*, 2008] by using four transcription factors (Oct4, Sox2, cMyc and Klf4) that are collectively referred to as Yamanaka-factors according to their discoverer. The iPSCs present exciting area of research with close linkage to regenerative medicine, transplantation medicine, drug development and disease modeling (see Figure 1). Moreover, the reprogrammed cells could be utilized to repair damaged tissues, since the transplant rejection could be avoided by generating patient-specific pluripotent stem cells.

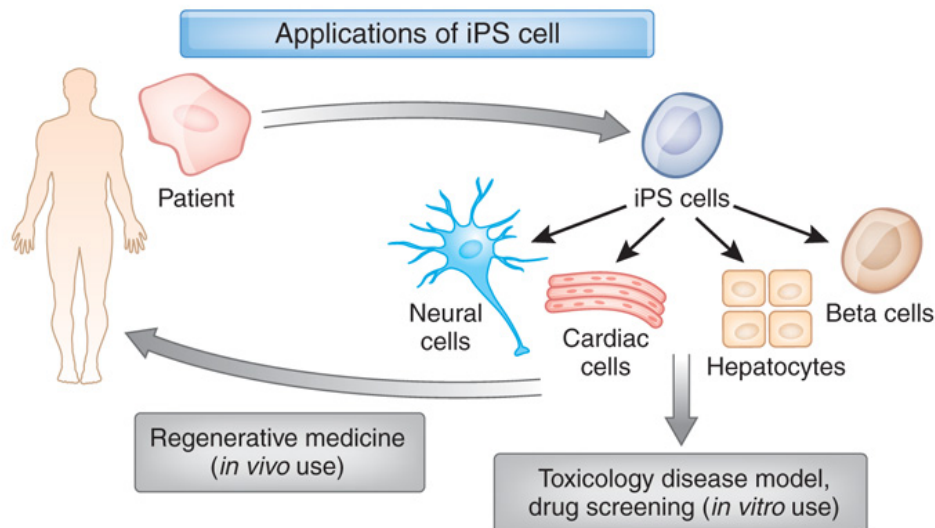


Figure 1. Usage of generated induced pluripotent stem cells as tools in transplant, drug and disease research [Yamanaka, 2009].

1.1.4 Asymmetrical division

Although possessing limitless division capacity, the stem cells are able to stay quiescent for long periods until going through cell division [What are adult stem cells?, 2012]. For maintaining the tissue homeostasis and generating differentiated progeny, the stem cells undergo process called *asymmetrical division* – a cell division where a stem cell produces one daughter cell remaining a stem cell and other specializing into specific cell fate. The asymmetrical division can be divided into two strategies. In the strategy based on divisional asymmetry, the dividing cell is already asymmetrical having the cell-fate determinants only on the other side of the cell, creating two dissimilar daughter cells: one inheriting the stem cell characters and other forced into differentiation fate. A recent study has demonstrated that when dividing asymmetrically, the subcellular contents of mammalian stem-like cells, such as mitochondria, will undergo selective apportioning: daughter cells maintain their stemness when receiving fewer old mitochondria [Katajisto *et al.*, 2015]. On the other hand, in environmental division, environmental influences guide the two generated initial similar daughter cells towards different pathways. [Alberts *et al.*, 2002] In addition, in cases such as injuries or damage, symmetrical stem cell division takes place in order to replace the lost stem cells [reviewed in Simons & Clevers, 2011].

1.1.5 Transit amplifying cells (TACs)

As mentioned, the stem cells give rise to a new stem cell and a progenitor for more specialized cells. The cells called *transit amplifying cells* (TACs) undergo transit amplification until achieving eventually the terminal differentiation. In comparison to infrequently dividing stem cells, TACs divide repeatedly, but do so only for a limited amount of division cycles. [Alberts *et al.*, 2002; reviewed in Simons & Clevers, 2011] TACs can be multipotent and capable of self-renewal [Mikkers & Frisen, 2005], but while they possess an essential role in tissue regeneration, serving as a precursor for differentiated cells, TACs are not stem cells as they are restricted in their regenerative potential.

1.1.6 Stem cell and niche

The balance between self-renewal and differentiation of the stem cells is crucial for accurate tissue renewal. What are the characteristics determining which progeny goes through transit amplification followed by terminal differentiation and which retains its stemness and exists as a stem cell? The local surrounding microenvironment, known as the *stem cell niche*, is an anatomical compartment controlling the stem cell behavior. Although identifying the stem cell niche properties has been challenging due to their complicated structures and lack of specific markers *in vitro*, remarkable information has been achieved in both mammalian and other model organisms.

The stem cell niches have been studied in mammalian tissues such as bone marrow [Zhang *et al.*, 2003; Calvi *et al.*, 2003], hair and skin follicle [Tumbar *et al.*, 2004; Clayton *et al.*, 2007], intestine [Barker *et al.*, 2007; Snippert *et al.*, 2010] and brain [Palmer *et al.*, 2000; Doetsch *et al.*, 1999]. Also, the studies regarding stem cell niche in *C. elegans* and *Drosophila* have provided important characteristics with respect to the stem cell niche function [Crittenden *et al.*, 2002; Xie & Spradling, 2000]. These studies have designated that the stem cell niches are as varied as their stem cell counterparts. Nevertheless, particular ‘hallmarks’ of the niches can be generalized.

First, the niche emanates extrinsic, environmental signaling pathways (such as Notch, FGFs, Hedgehog, Wnts or BMPs) that regulate self-renewal and determine the stem cell fate and number. It should be noted, however, that these signaling pathways might differ between various tissues. Also, the stem cell behavior is regulated by physical attachment to basal lamina or supporting cells since the niche functions as an ‘anchor’

for the stem cells. This keeps the stem cells in close vicinity to other cells by adherens junctions and it might be essential for holding the stem cells within the niche and remaining undifferentiated. Thirdly, a recent study demonstrates that the relative stiffness and elasticity of the microenvironment effects on the stem cell fate and might guide the stem cells towards different specialization [Engler *et al.*, 2006]. Furthermore, an asymmetric structure of the stem cell niche can provide positional cues for cell identity: in the case of the intestinal epithelium, undifferentiated stem cells are situated in the bottom of the intestinal crypt whereas the TACs migrate upwards and experience changes in the Wnt and BMP niche signals. The niche structure provides also a shelter from extrinsic damage. [Reviewed in Jones & Wagers, 2008; reviewed in Li & Xie, 2005]

1.2 Intestinal stem cells (ISCs) and intestinal maintenance

1.2.1 Intestinal stem cells

The lining of small intestine undergoes self-renewal every 4 to 6 days, having one of the most rapid turnover of adult tissues. This renewal of intestinal epithelium is maintained by *intestinal stem cells* (ISCs) located in the bottom of intestinal crypts (the crypts of Lieberkühn). Structure of the small intestine consists pocket-like crypts located around digit-like villi (see Figure 2). Each villus receives differentiated cells from several ambient crypts.

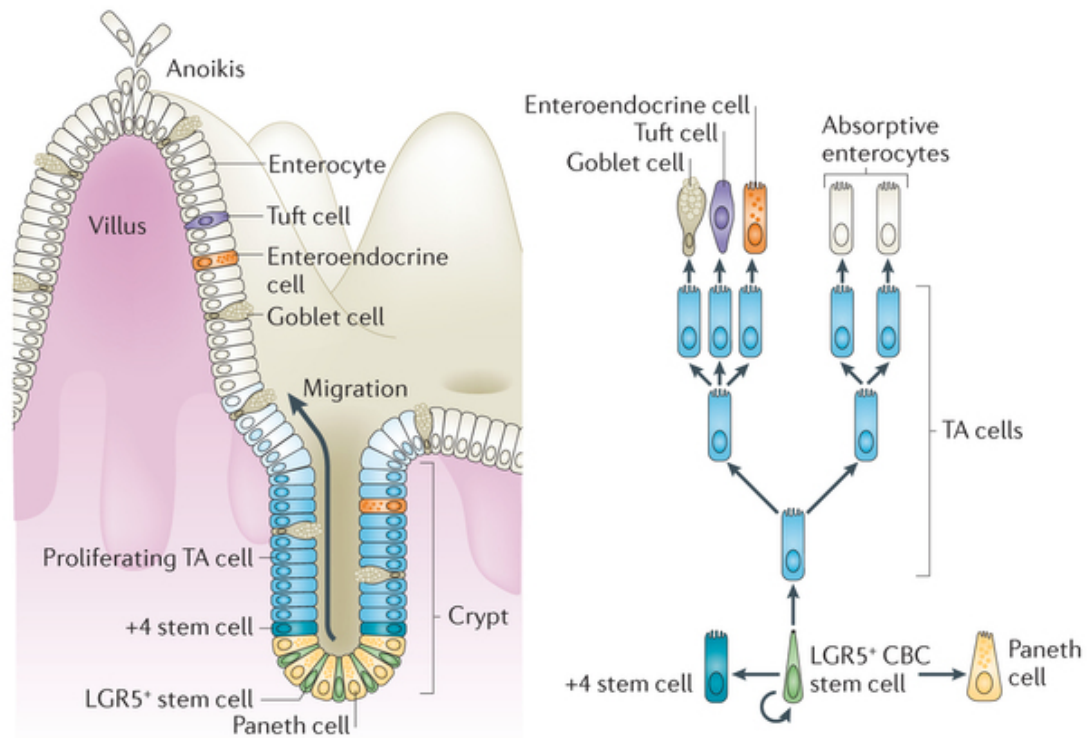


Figure 2. Intestinal stem cells terminally differentiate into various functional cells while migrating towards the villus tip [adapted from Barker, 2014].

The main function of small intestine is to secrete digestive enzymes in order to break down the proteins and other molecules of food, and to absorb digested nutrient molecules and minerals. The villi enlarge the epithelium of the small intestine, providing effective absorption of the nutrients from the lumen. The ISCs, also called crypt base columnar (CBC) cells, give rise to rapidly dividing TACs (4-5 cell division rounds), which eventually differentiate into four main cell types: nutrient-absorbing enterocytes, secretory goblet cells, enteroendocrine cells and Paneth cells (see Figure 2). The first three cell types migrate from the crypt towards the top of the villi, undertaking the digestive and absorptive functions of the intestine, and finally, die at the villus tips after 2-3 more days through apoptosis [Snippert *et al.*, 2010]. Contrary to actively cycling ISCs, the quiescent '+4 positioned' stem cells (directly above the Paneth cells) operate as reserve stem cells [Li & Clevers, 2010] that can be recruited back into the stem cell niche to regain a lost ISC, for instance, upon damage. Paneth cells, the fourth differentiated ISC progeny, migrate downwards and locate in the bottom of the crypts interspersed between ISCs [Barker *et al.*, 2007]. The intestinal crypt contains approximately 5-15 Paneth cells [Bry *et al.*, 1994]. Furthermore, the pentagon-like shape of Paneth cells provides maximal shared membrane with the small, triangle-like shaped ISCs,

suggesting that Paneth cells are in close physical association with ISCs [Sato *et al.*, 2011; Snippert *et al.*, 2010]. Indeed, the terminally differentiated Paneth cells produce regulatory molecules such as growth factors, antimicrobial peptides and digestive enzymes [Bry *et al.*, 1994] and provide signals (e.g. epidermal growth factor, Wnt and Notch) essential for the ISC maintenance [Sato *et al.*, 2011; reviewed in Porter *et al.*, 2002]. Due to direct physical interaction with the ISCs, the Paneth cells may be referred as the probable main controllers of ISC number. Moreover, several recent studies [Shroyer *et al.*, 2005; Mori-Akiyama *et al.*, 2007; Bastide *et al.*, 2007; Sato *et al.*, 2011] have presented that reducing the Paneth cell number will also decrease the number of stem cells.

Recent studies have demonstrated that ISCs can be characterized by high expression of Wnt target gene *Lgr5* (leucine-rich G protein-coupled receptor 5) and that each murine intestinal crypt contains approximately 16 intestinal *Lgr5* stem cells [Barker *et al.*, 2007; Snippert *et al.*, 2010]. The *Lgr5*-ISCs divide approximately once a day and they receive niche support from their more differentiated progeny [Barker *et al.*, 2007; Sato *et al.*, 2011]. However, since the intestinal regeneration and the intestinal crypt homeostasis are maintained by the ISC proliferation, particular signaling pathways need to be examined and understood more precisely.

1.2.2 Maintenance of intestinal crypt homeostasis

What are the factors regulating the intestinal stem cell homeostasis and which molecular niche signals drive the ISCs towards differentiation? For ensuring the intestinal crypt homeostasis, each ISC should divide forming a new stem cell and a TAC. Indeed, the homeostasis of the crypts results from neutral competition (number of *Lgr5* stem cells in a crypt determined by the available Paneth cell surface) between *Lgr5* stem cells [Barker *et al.*, 2007; Snippert *et al.*, 2010] and also, the stem cell fate might not be definitively determined in division [Ritsma *et al.*, 2014]. Nonetheless, a number of studies have indicated the importance of niche signaling pathways that possess essential role when controlling the balance between self-renewal and differentiation of ISCs.

Shape of the intestinal crypt and the positions of various cell types inside the crypt arises questions whether the niche signaling fluctuates among the crypt. As disclosed previously, the Paneth cells have been indicated to provide essential niche signals for their progenitor *Lgr5* stem cells [Sato *et al.*, 2011].

The Notch signaling, provided by ligands located on the surface of the Paneth cells, is required to preserve Lgr5 stem cells in an undifferentiated state and to maintain proliferating crypt progenitors in the intestinal epithelium. Cell-to-cell Notch signaling between Paneth cells and Lgr5 stem cells highlights the importance of direct physical contact. Indeed, when losing the contact with the Dll1/4-expressing Paneth cells and exiting Paneth cell zone, the stem cells differentiate and migrate upwards along the crypt walls. The Notch signaling is proved to be essential for Lgr5 stem cell survival since the inhibition of the Notch pathway results in stem cell loss. [van Es *et al.*, 2005; Fre *et al.*, 2005; van Es *et al.*, 2010; VanDussen *et al.*, 2012; reviewed in Clevers, 2013]

Similarly to the Notch pathway, the Wnt signaling is also provided by Paneth cells. The short-range Wnt maintains stem cell fate and drives the proliferation of ISCs and the terminal differentiation of Paneth cells. [Reviewed in Sato & Clevers, 2013] The Wnt has also been indicated as the driver of crypt proliferation and the Wnt signals are crucial for the establishment of the stem cell zone. Furthermore, the Wnt agonist R-spondin-1 (the ligand of Lgr5), among all RSPOs, is strong modulator of the Wnt pathways and might enhance Wnt signals. [Reviewed in Koo & Clevers, 2014; Sato *et al.* 2011; reviewed in Clevers, 2013] Only the direct neighbor cells of Paneth cells receive strong Wnt signals [Sato *et al.*, 2011], implicating that the Wnt levels are rapidly decreasing when migrating upwards the crypt walls.

Whereas the Wnt gradient decreases upwards the crypt, the BMP (bone morphogenetic protein) gradient functions vice versa. The BMP-signaling pathway through β -catenin regulates the crypt negatively and suppresses the Wnt signaling, ensuring the balanced stem cell self-renewal [Haramis *et al.*, 2004; He *et al.*, 2004]. The activity of BMP can be regulated by its own inhibitor, Noggin, that induces an expansion of crypt numbers and is required when culturing intestinal organoids *in vitro* [Sato *et al.*, 2009; Haramis *et al.*, 2004]. However, sources of both Wnts and BMPs remain still uncertain.

As a conclusion, the nature of the neighboring cells is proved to be determining extrinsically the intestinal stem cell fates of individual progeny. The specific signaling pathways discussed above are playing a key role when maintaining the tissue homeostasis and regeneration of the intestinal crypts. Since multiple regulatory signals exist *in vivo*, it is essential to provide this 'signaling cocktail' also when culturing intestinal organoids

in vitro in order to achieve proper regulation of stem cell self-renewal. In addition, the importance of ECM should not be neglected (see chapter 1.3).

1.2.3 *In vitro* culture of intestinal organoids

Since being the most rapidly self-renewing mammalian tissue, the small intestine and the ISCs provide suitable basis to study adult intestinal stem cells *in vitro*. Even though stem cells have been cultured in two-dimensional (2D) conditions with promising results, it is not possible to study and understand the stem-cell-driven crypt-villus biology if the ISCs are not cultured in *in vivo* reminiscent environment. Thus, the recent advancements demonstrating that single Lgr5-ISCs can be successfully isolated and cultured *in vitro* in three-dimensional (3D) culture that mimics their original *in vivo* niche conditions [Sato *et al.*, 2009] are of great interest.

The cultured ISCs grow into complex 3D *intestinal organoids*, also called as *mini-guts*, that are fully self-organizing in the absence of any mesenchymal components. In order to preserve this *in vivo* reminiscent epithelial structure, the isolated Lgr5-ISCs require a 3D laminin- and collagen-rich matrix (Matrigel, BD Biosciences) substrate that mimics the basal lamina and supports the intestinal epithelial growth. In addition, a cocktail of growth factors (EGF, the BMP inhibitor Noggin and R-spondin) is essential for the stem cell maintenance. The organoid formation begins when a bud forms around the isolated ISC. Within 3 days, the bud develops into a crypt-like structure consisting both stem and Paneth cells. In these crypt-like buds, Paneth and stem cells are pushed outwards from the central lumen whereas the proliferating TACs lose contact with the Wnt and Notch signal providing Paneth cells and are mechanically pushed towards the lumen by younger TACs. When experiencing decreasing levels of Wnt since losing the contact with Paneth cells, the TACs undergo terminal differentiation into one of the villus epithelial cell types (eg, enterocytes, goblet cells, enteroendocrine cells). Indeed, the *in vitro*-generated organoids consist of a central lumen flanked by a villus epithelium (villus domain) and multiple crypt-like structures (crypt domain) projected outwards from the lumen. [Sato *et al.*, 2009; Sato *et al.*, 2011; reviewed in Koo & Clevers, 2014; reviewed in Sato & Clevers, 2013]

The importance of Paneth cells in ISC maintenance was observed when culturing intestinal organoids *in vitro*. The ever-expanding intestinal organoids can be generated efficiently only when the Lgr5-ISCs are in reassociation with the Paneth cells. Further-

more, the Lgr5-ISCs are critically dependent on Notch and Wnt signals, also provided by the Paneth cells. Indeed, Paneth cells possess a key role when establishing organoids from single Lgr5-ISCs *in vitro*. [Sato *et al.*, 2011; Sato *et al.*, 2009] It has also been demonstrated that, *in vitro*, cell-cell doublets between stem and Paneth cell robustly generated intestinal organoids, while single sorted Lgr5-ISCs rarely survived [Yilmaz *et al.*, 2012].

1.3 Extracellular matrix (ECM)

In order to communicate and regulate the stem cell function, the signaling between cells is not the only way. The extracellular matrix (ECM), secreted locally by the cells, is composed of fibrous matrix proteins (such as collagens, elastins, fibronectins and laminins) and heavily glycosylated proteoglycans. The structural proteins of ECM provide attachment sites for stem cells, which anchor to ambient matrix through integrins. The physical composition of ECM provides tissues their strength and elasticity, and it might have a critical role when regulating the stem cell function. [Reviewed in Pentimikko & Katajisto, 2014] Moreover, since being the key component of the stem cell niche, the ECM might influence cell fate choices [Hall & Watt, 1989]. Key unanswered questions are how stem cells sense and respond to signals provided by the ECM and how the ECM regulates stem cell fate.

1.3.1 Composition of ECM

Composition of the ECM varies between and within tissues and different cell types interact with the ECM in several ways. The necessary features of tissue, such as tenacity, elasticity and stiffness, result from characteristics of ECM's molecule structure. Macromolecules of the ECM can be roughly divided into three groups – fibrils forming proteins, glycoproteins of the matrix and proteoglycans. Even though the ECM is present in each tissue, its significance is emphasized in connective tissues.

The connective tissue is formed around large protein filaments, which are mainly created by the collagens found in fibrous tissues. Collagens are proteins formed of three subunits and at least one part of the protein forms a triple helix. The fibrous collagen gives the ECM its distinctive tensile strength. Type I collagen is quantitatively the most abundant in mammals, being the main component in bone, tendon and loose connective

tive tissue. Some collagens, such as type IV collagen that is the key component of basement membrane, form mesh instead of filaments. [Heino & Vuento, 2014] Equally to collagens, laminins are wide group of genes that are formed of three subunits (called α , β and γ) bound together creating cross-like proteins. Laminins have the ability to attach into each other, constituting a wide 3D mesh that fulfills both layers of basement membrane. In addition, laminins provide anchoring sites to epithelial cells. [Reviewed in Frantz *et al.*, 2010]

Fibronectins are glycoproteins that bind to collagens and receptor proteins called integrins. Moreover, fibronectins have the ability to attach into large number of other molecules, such as other fibronectins and certain proteoglycans and glycoproteins. The cell-ECM adhesion is enabled via fibronectins and cells are capable to migrate along the surface coated by the fibronectin molecules. Receptors on the cell surface can adhere to several parts in fibronectin and this provides the variety in binding opportunities. Along with the fibronectins, there are several other ECM proteins that have the ability to adhere to other molecules. Tenascins and thrombospondins are glycoproteins that bundle functional protein complexes; furthermore, they have a role in filament formation of connective tissue and might act as cell adhesion inhibitor proteins. [Heino & Vuento, 2014; reviewed in Frantz *et al.*, 2010]

Elastins and fibrillins are elastic proteins that have a role in regulating the elasticity of the ECM in a given tissue. The elastin proteins bind to each other with covalent cross-bonds and form filaments. Since being highly sustainable molecules, the elastin filaments renew infrequently. Altogether, these fibrous proteins (collagen, laminin, fibronectin and elastin) have both adhesive and structural functions in the ECM. [Reviewed in Watt & Huck, 2013; reviewed in Frantz *et al.*, 2010]

Integrins, also called as *cell adhesion molecules*, are integral proteins of cell membrane. The cell-ECM adhesions are controlled by the integrins binding to the ECM. Integrins are heterodimers (consist one α - and β -subunit) that interact with various regulatory proteins [Ferreira *et al.*, 2009; Hynes, 2002] and the focal adhesions are formed by integrin-mediated adhesions to the ECM. There is a wide range in properties of integrins, therefore they can interact with various growth factor receptors and regulatory proteins. Most of the integrins are expressed in various cell types and also, various cell types express several different integrins. When having multiple attachment sites to the ECM's proteins, cells can probe the ambient environment without losing the touch. Fi-

nally, the intracellular signaling pathways are controlled by the integrins, designating their importance in the ECM.

A special form of the ECM, the basement membrane, is a thin non-cellular tissue that separates the outer (epithelial, mesothelial or endothelial) tissue from underlying connective tissue. It provides attachment surface to epithelial cells and anchors the epithelium to its loose connective tissue through substrate adhesion molecules. Type IV collagen and laminin are typical molecules of the basement membrane. [Heino & Vuento, 2014]

1.3.2 Functions of ECM

The key function of the ECM is to form a supporting framework, operating as a mechanical support in tissues and to protect the cells by physically, chemically and immunologically. It provides an organized environment to cells and it controls the cell functions such as proliferation, differentiation, migration and polarization. Moreover, the matrix has an active role in regulating the cell behavior, survival, development and function. As a conclusion, the ECM has specific functional requirements depending on the tissue and it interacts with cells both mechanically and chemically. Through a process called mechanotransduction, cells sense mechanical cues from the ECM and act in a proper manner, for example changing the cell shape and size. The anchorage dependence explains the urgent need of ECM to cells in order to grow, proliferate and survive. *In vitro* cell culture has indicated that when spread over a large matrix surface area, the cells are more likely to survive and proliferate than when cultured in a single patch [Chen *et al.*, 1997]. Furthermore, the cell spreading and the cell's cytoskeleton organization could be influenced by the organization of the ECM.

1.3.3 Stiffness of ECM

In a large extent, the physical properties of tissues, such as elasticity or tenacity, are defined by the ECM's molecular characteristics. When scrutinizing the stiffness of the ECM, the limits of strength and flexibility are determined by the collagen filaments in given tissue. Moreover, the fibrin fibrils and fibronectins define the mechanical properties of the ECM. [Reviewed in Watt & Huck, 2013]

Disease and age are factors influencing the tissue stiffness *in vivo*, and in general, the stiffness is far lower compared to *in vitro* culture circumstances [reviewed in Lu *et al.*, 2012]. This places challenges when mimicking the right ECM conditions: stem cell populations response in different ways when varying the bulk stiffness. Since the tissue stiffness changes during time provoking changes in stem cell behavior *in vivo*, the stem cell differentiation and behavior could be studied by regulating the matrix composition in 3D culture.

Several recent studies have demonstrated that the stiffness of the ECM influences stem cell differentiation [reviewed in Watt & Huck, 2013; reviewed in Lu *et al.*, 2012]. Mesenchymal stem cells were directed into bone differentiation in stiff environments, whereas soft substrates drove the cells into adipocyte differentiation [Engler *et al.*, 2006]. Corresponding observations have been made with skeletal muscle stem cells [Gilbert *et al.*, 2010] and with adult neural stem cells [Saha *et al.*, 2008]. Furthermore, wide range of model substrates (including such as collagen and hyaluronic acid gels, polymer networks and electrospun nanofibres) have been used in order to examine the influence of stiffness [Saha *et al.*, 2008; Hadjipanayi *et al.*, 2009; Jha *et al.*, 2011].

Even though there is a number of studies in terms of matrix stiffness and 3D culture with mammalian stem cells, the significance of ECM stiffness in intestinal stem cell niche is still undetermined. As stated previously, the intestinal organoids that resemble the *in vivo* intestinal crypt conditions have been successfully cultured in 3D environment [Sato *et al.*, 2009]: this could be utilized when researching the stature of ECM stiffness particularly in regulation of intestinal stem cell function.

2 Objectives

Even though the intestinal cells have been widely studied in 2D cell culture during recent years, the *in vitro* culturing environments have not resembled sufficiently the original *in vivo* intestinal niche conditions. For further conclusions, the ISCs should be cultured in conditions that provide more accurate microenvironmental cues and mechanical structure. Since a multitude of factors maintaining the intestinal crypt homeostasis (the fibrous structural proteins that form the mechanical structure of the ECM, and the growth factors regulating stem cell function and proliferation) are present *in vivo*, it is indispensable to express them also when cultured *in vitro*.

Intestinal organoids have been successfully cultured in Matrigel-based 3D matrices during past years and it has been demonstrated that the isolated ISCs grow into 3D intestinal organoids that mimics the structure of original *in vivo* intestinal crypts. Nevertheless, the precise significance of the ECM stiffness in intestinal stem cell function has not been studied.

The aim of this thesis was to study whether the stiffness of extracellular matrix has an influences intestinal stem cell function when cultured in three-dimensional culture. In order to carry out this study, murine intestinal crypts containing Lgr5+ stem cells were isolated and cultured in Matrigel-based 3D matrix. The variations in the ECM stiffness were modified by generating different concentrations of Matrigel (from 50% to 90%). It was also tested whether the matrix stiffness could be increased by cross-linking Matrigel with calcium alginate suspension.

3 Materials and methods

3.1 Isolation and culture of intestinal crypts

Crypt culture medium

The intestinal crypts and organoids were cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 (Advanced DMEM/F12, Gibco) basal medium supplemented with Recombinant Human epidermal growth factor (rhEGF, R&D Systems), Recombinant Murine Noggin (PeproTech), Recombinant Mouse R-Spondin 1 (R&D Systems), N-Acetyl-L-cysteine (NAC, Sigma), N-2 Supplement (Gibco), B-27® Supplement (Gibco) and GlutaMAX™ (Gibco).

The supplements were diluted into 10 ml aliquot of DMEM/F12 medium. The used supplement quantities are presented in Table 1:

Table 1. Medium and supplement details of the crypt culture medium.

Medium	Manufacturer	Concentration (µl/ml)	Used quantity (ml)
Advanced DMEM/F12	Gibco		10 ml

Supplement	Manufacturer	Concentration (µl/ml)	Used quantity (µl)
rhEGF	R&D Systems	0.1	1
Noggin	PeproTech	0.2	2
R-Spondin 1	R&D Systems	2	20
NAC	Sigma	1	10
N-2	Gibco	10	100
B-27	Gibco	20	200
Glutamax™	Gibco	10	100

The crypt culture medium could be stored in +4°C for 7 days without losing its function.

Isolation

Selected mouse was sacrificed with CO₂ and cervical dislocation. Small intestine was removed from the mouse and kept in ice cold phosphate buffered saline (PBS) while proceeding the following steps. Aliquot of Matrigel was taken on ice for melting.

First, fat and mesentery were carefully cleaned away and lumen was washed with ice cold PBS until the intestine appeared white or pink. The intestine was opened longitudinally and to remove mucus, the intestine was gently rubbed with fingers against petri dish in cold PBS. The small intestine was carefully cut into small 1 mm fragments. The fragments were then placed into a 50 ml conical tube and the tube was filled with 30 ml of ice cold 10 mM PBS-EDTA. The tube was incubated on ice for 45 min, intermittently shaking. The supernatant was replaced and the foam formed on top was discarded at least 3 times during the incubation. Discarding the formed top foam with a pipette was essential. After the incubation, the fragments were resuspended one more time with 30 ml of ice cold 10 mM PBS-EDTA and incubated again on ice for 60 min, intermittently shaking.

After that, the sample was triturated with a 25 ml pipette for 3 times. To remove villus material and tissue fragments, the sample was poured through a 70 μ m filter into a new 50 ml conical tube. At this point, the flow was mainly free crypts; the amount of the crypts could be ensured by microscoping a small aliquot. The sample was centrifuged at 300 g at RT for 5 min. To remove EDTA from the sample, the supernatant was carefully discarded and the crypt pellet was diluted with 5 ml of ice cold PBS. The sample was placed into a 15 ml conical tube and centrifuged again at 300 g at RT for 5 min. The supernatant was carefully discarded and the crypt pellet was resuspended in 70-200 μ l of the crypt culture media, dependent on the pellet size.

Finally, the crypts were plated into 48-well-plate in 20-30 μ l drops in desired concentrations of Matrigel and PBS, depending on the current experiment. The wells next to the plate edge were not used. The concentrations used in individual experiments are described in chapters 3.2 and 3.3. The plate was incubated at +37°C for 30 min to let Matrigel to solidify and after that, the wells were overlaid with 350 μ l of the crypt culture media. To maintain the necessary humidified atmosphere, 350 μ l of sterile deionized water was pipetted to the surrounding wells. The crypt culture media was changed in every 2-3 days on the grounds of the growing speed of the crypts.

Subculture

Growing organoids were subcultured after 7-8 days of plating to prevent the organoids from overgrowing. First, the organoids were collected with Matrigel and the crypt culture media to a 15 ml conical tube. All collateral wells from each concentration were

gathered together. The tubes were centrifuged at 100 g at RT for 5 min to get all the organoids fall to the bottom of the tube. The extra media were gently aspirated and the organoids were left approximately in 200 μ l volume.

To break the organoids to single crypts, the samples were then triturated 40 times with 200 μ l pipette. The detaching of the crypts could be ensured by microscoping a sample and continuing the trituration if needed. 4 ml of sterile PBS were added to each tube and the tubes were centrifuged again at 100 g at RT for 1 min. Extra PBS was aspirated away as much as possible and the crypt pellets were resuspended in 5-10 μ l of the crypt culture media. The tubes were placed on ice.

Finally, the crypts were plated into a 48-well-plate in 20-30 μ l drops in desired concentrations of Matrigel and PBS, depending on the current experiment. The wells next to the plate edge were not used. The plate was incubated at +37°C for 30 min to let Matrigel to solidify and after that, the wells were overlaid with 350 μ l of the crypt culture media. To maintain the necessary humidified atmosphere, 350 μ l of sterile deionized water was pipetted to the surrounding wells.

3.2 Modifications of Matrigel-based culture

The examination of extracellular matrix stiffness and its effects on intestinal stem cell function was executed by using BD Matrigel Basement Membrane Matrix (BD Matrigel Matrix Growth Factor Reduced, Phenol Red-Free, BD Biosciences) as a main component in 3D matrix culture of intestinal crypts and organoids. Matrigel is a laminin- and collagen-rich matrix that can be utilized when culturing stem cells in 3D culture.

In the experiments based on Matrigel-modifications (5 rounds), the main purpose was to study whether the concentration of used Matrigel affected the function and the qualities of the growing crypts and organoids. In these experiments, the matrix stiffness varied between 250-500 Pa [Soofi *et al.*, 2009].

After the isolation of the intestinal crypts, the first step was to prepare the desired concentrations of Matrigel. The quantities of used components for each Matrigel concentration (50%-90%) are presented in Table 2:

Table 2. Quantities of used components for Matrigel concentrations.

Component	Used quantities (µl) for Matrigel concentrations				
	50%	60%	70%	80%	90%
Matrigel	40	48	56	64	72
PBS	32	24	16	8	0
Crypts	8	8	8	8	8
Total volume (µl)	80	80	80	80	80

The melted Matrigel aliquots, PBS and the isolated crypts were kept on ice while proceeding the following steps; it was necessary to prevent Matrigel aliquots from solidifying. Each of the concentrations were prepared into own 1.5 ml eppendorf tube and kept on ice. First, the desired quantities of Matrigel and ice cold PBS (see Table 2) were pipetted to marked eppendorfs and components were mixed together carefully to obtain homogenous mixtures. Then, the isolated crypts were added to mixtures and once again mixed carefully.

Finally, the crypts were plated into a 48-well-plate in 20 µl drops, 3 collateral wells per each concentration. The wells next to the plate edge were not used. The plate was incubated at +37°C for 30 min to let Matrigel to solidify and after that, the wells were overlaid with 350 µl of the crypt culture media. To maintain the necessary humidified atmosphere, 350 µl of sterile deionized water was pipetted to the surrounding wells.

The subculture of the growing organoids was executed according to the protocol (see chapter 3.1) and the protocol for microscopic analysis and quantifications/counting is presented in chapter 3.4. Results from the experiments based on the Matrigel-modifications are presented in chapter 4.1.

3.3 Modulations of stiffness via cross-linking the matrix

In the experiments based on cross-linking Matrigel with calcium and alginate (3 rounds), the main purpose was to increase the stiffness of the ECM even more by cross-linking Matrigel with calcium and alginate. The ECM stiffness in these cross-linking experiments varied between 100-1200 Pa [Chaudhuri *et al.*, 2014].

Solutions in phases 1, 2 and 3 were prepared in the beginning of the first round and they could be utilized during the rest of the rounds (after vortexing properly); mixtures in phases 4 and 5 were prepared afresh in every round. The solutions from phases 2 and 3 were stored in +4°C and the solution from phase 1 were stored in RT without losing their function.

Phase 1

In phase 1, calcium sulfate dihydrate ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, Sigma) powder was dissolved into 50 ml of sterile MQ water in order to prepare 1.22 M calcium sulfate (CaSO_4) solution. The required quantity of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ was counted by using the formula 1:

$$m = cVM \quad (1)$$

where	m	is the required mass of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (g)
	c	is the desired concentration of CaSO_4 (mol/l)
	V	is the volume of MQ water (l)
	M	is the molecular weight of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (g/l)

$$m = cVM = 1.22 \text{ mol/l} * 0.05 \text{ l} * 172.17 \text{ g/mol} = 10.50237 \text{ g} \approx 10.50 \text{ g}$$

Phase 2

Next step was to prepare 40 mM, 80 mM and 160 mM concentrations of CaSO_4 solution prepared in phase 1. Desired concentrations of CaSO_4 were prepared by diluting 1.22 M CaSO_4 into DMEM/F12 medium. The required quantities of 1.22 M CaSO_4 and DMEM/F12 were counted by using the formulas 2 and 3:

$$c_1 * V_1 = c_2 * V_2 \quad (2)$$

$$V_1 = \frac{c_2 * V_2}{c_1}$$

where

c_1	is the concentration of CaSO_4 (mol/l)
V_1	is the volume of CaSO_4 (l)
c_2	is the desired CaSO_4 concentration (mol/l)
V_2	is the total volume of CaSO_4 concentration (l)

$$V_3 = V_2 - V_1 \quad (3)$$

where

V_1	is the volume of CaSO_4 (l)
V_2	is the total volume of CaSO_4 concentration (l)
V_3	is the volume of DMEM/F12 (l)

Counted the required volume of 1.22 M CaSO_4 for 40 mM solution:

$$V_1 = \frac{c_2 * V_2}{c_1} = \frac{0.04 \text{ mol/l} * 0.002 \text{ l}}{1.22 \text{ mol/l}} = 0.0000655 \text{ l} = 0.0655 \text{ ml} = 65.5 \mu\text{l}$$

Counted the required volume of DMEM/F12 for 40 mM solution:

$$V_3 = V_2 - V_1 = 0.002 \text{ l} - 0.0000655 \text{ l} = 0.0019345 \text{ l} = 1.9345 \text{ ml}$$

The required quantities for 80 mM and 160 mM CaSO_4 concentrations were counted similarly. The quantities of used components for each CaSO_4 concentration (40 mM - 160 mM) are presented in Table 3:

Table 3. Quantities of used components for CaSO_4 concentrations.

Component	Used quantities (ml) for CaSO_4 concentrations		
	40 mM	80 mM	160 mM
1.22 M CaSO_4	0.0655	0.1311	0.2622
DMEM/F12	1.9345	1.8689	1.7378
Total volume (ml)	2	2	2

Each of the concentrations were prepared into own 15 ml conical tube and kept on ice. First, the desired quantities of DMEM/F12 were pipetted into marked conical tubes. Then, the desired quantities of 1.22 M CaSO_4 were added to tubes and components were mixed together carefully to obtain homogenous solutions. The tubes were kept on ice.

Phase 3

In phase 3, 0.25 g of alginic acid sodium salt (Sigma) was dissolved into 10 ml of DMEM/F12 medium in a 15 ml conical tube to obtain 2.5% alginate concentration. The solution was mixed properly to obtain homogenous solution and kept on ice.

Phase 4

2.5% alginate solution was combined with Matrigel in order to prepare an alginate Matrigel mixture (1 part of alginate solution, 3 parts of Matrigel). The isolated crypts were added to the alginate Matrigel mixture in a 10% volume of total mixture volume. The actual quantities of used components are presented in Table 4:

Table 4. Quantities used for 1:3 alginate Matrigel mixture.

Component	Used quantities (µl)
	2.5% alginate Matrigel mixture (1:3)
2.5% alginate solution	150
Matrigel	390
Crypts	60
Total volume (µl)	600

Phase 5

Finally, the desired CaSO_4 concentrations (10 mM – 40 mM) were prepared by diluting the 40 mM – 160 mM CaSO_4 concentrations to alginate matrigel mixture prepared in phase 4. 0 mM CaSO_4 concentration was prepared by using DMEM/F12. The actual quantities of used components are presented in Table 5:

Table 5. Quantities used for final CaSO_4 concentrations.

Component	Used quantities (μl) for final CaSO_4 concentrations			
	0 mM	10 mM	20 mM	40 mM
DMEM/F12	30			
40 mM CaSO_4		30		
80 mM CaSO_4			30	
160 mM CaSO_4				30
Alginate Matrigel mixture	90	90	90	90
Total volume (μl)	120	120	120	120

50% Matrigel concentration mixture was used as a 0 mM control mixture. The quantities of used components are presented in Table 6:

Table 6. Quantities used for 0 mM control mixture.

Component	Used quantities (μl) for control mixture
	0 mM (50% Matrigel)
Matrigel	60
DMEM/F12	12
Crypts	48
Total volume (μl)	120

Each of the concentrations were prepared into own 1.5 ml eppendorf tube and kept on ice. First, 30 μl of original CaSO_4 concentrations or DMEM/F12 were pipetted to tubes. 90 μl of alginate Matrigel mixture were added to the tubes and triturated properly. 0 mM control mixture (50% Matrigel) was prepared similarly to the concentrations in the experiments based on Matrigel-modifications.

Finally, the samples were plated into a 48-well-plate in 30 μl drops, 3 collateral wells per each concentration. The wells next to the plate edge were not used. The plate was incubated at +37°C for 30 min to let Matrigel to solidify and after that, the wells were overlaid with 350 μl of the crypt culture media. To maintain the necessary humidified atmosphere, 350 μl of sterile deionized water was pipetted to the surrounding wells.

The subculture of the growing organoids was executed according to the protocol (see chapter 3.1) and the protocol for microscopic analysis and quantifications/counting is presented in chapter 3.4. Results from the cross-linking experiments are presented in chapter 4.2.

3.4 Microscopic analysis and quantifications

Day 1

For analysis of started crypts, the started crypts were counted next day (day 1) after isolating and culturing the intestinal crypts. The started crypts were counted separately from each collateral well using Nikon Eclipse TS100 microscope equipped with Nikon Plan Fluor 4x/0.13, Nikon 10x/0.25 Ph1 ADL and Nikon LWD 20x/0.40 Ph1 ADL objectives. The data was collected and tabled after counting. If the crypts were isolated and cultured on Friday, the counting of started crypts was performed on next Monday (day 3). A started crypt could be identified from dead or broken crypt by its roundish shape and dark outline (see Figure 3).

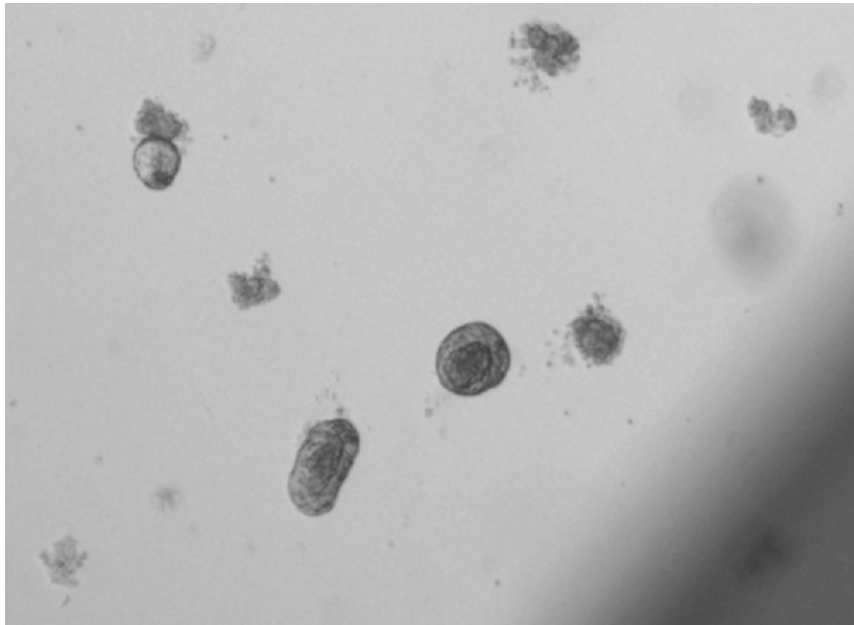


Figure 3. Growing small intestinal crypts imaged on day 1.

Day 4

The wells were imaged after 4 days of culturing. The 5x images of started crypts were acquired using Leica DM IRB microscope equipped with Leica DC 300F camera. The objective used was Leica Germany 5x. From each collateral well, 4-5 images were taken.

Day 7

After 7 days of culturing, the wells were imaged for analysis of crypt widths. The 10x images of the growing organoids were acquired using Leica DM IRB microscope equipped with Leica DC 300F camera. The used objectives were Leica Germany 5x, Leica Germany HC PL Fluotar 10x/0.30 PH 1 and Leica Germany 20x PH 2. All growing organoids from each collateral well were imaged. The diameters of the crypts were measured from 10x images using the ImageJ program (version 1.47v, National Institute of Health). The diameter was measured from the bottom of the crypt (see Figure 4).



Figure 4. Measurement line for crypt width analysis.

For analysis of paneth cell quantity in single crypt, the paneth cells were counted also after 7 days of culturing. The paneth cell quantity per crypt was counted separately from each collateral well using Nikon Eclipse TS100 microscope equipped with Nikon Plan Fluor 4x/0.13, Nikon 10x/0.25 Ph1 ADL, Nikon LWD 20x/0.40 Ph1 ADL and Nikon LWD 40x/0.55 Ph1 ADL objectives.

Also, since the subculturing of the growing organoids was (in most cases) performed on day 7, 5x images of splitted organoids were taken after the subculture. The images were acquired using Leica DM IRB microscope equipped with Leica DC 300F camera and Leica Germany 5x objective. 4-5 images were taken from each collateral well.

Day 9

For analysis of crypt quantity in single organoid, the number of crypts in single organoid was counted after 9 days of culturing and 2 days of subculturing. The crypts were counted from each growing organoid, separately from each collateral well. The data was acquired using Nikon Eclipse TS100 microscope equipped with Nikon Plan Fluor 4x/0.13, Nikon 10x/0.25 Ph1 ADL, Nikon LWD 20x/0.40 Ph1 ADL and Nikon LWD 40x/0.55 Ph1 ADL objectives.

4 Results

4.1 Intestinal organoid culture in Matrigel bio-matrix with variable concentrations

The isolated intestinal crypts were cultured in five separate Matrigel concentrations, in order to mimic the variable ECM stiffness found in the body. The key factor changing between the concentrations was the amount of laminin, due to the variations in Matrigel percentage. The number of started crypts, the number of Paneth cells in single crypt and the number of crypts in single organoid were under examination. Moreover, the widths from single crypts were measured.

The started crypts were counted on the next day after each isolation and culture, gathering the results altogether from five rounds of experiments (Figure 5). The results from each round were normalized to the average of that given experiment in order to compensate for the variability between crypt preps. The individual data points are represented as blue dots above each concentration. This data suggests that the isolated intestinal crypts started to grow and eventually form organoids more likely in loose matrix compared to stiffer matrix.

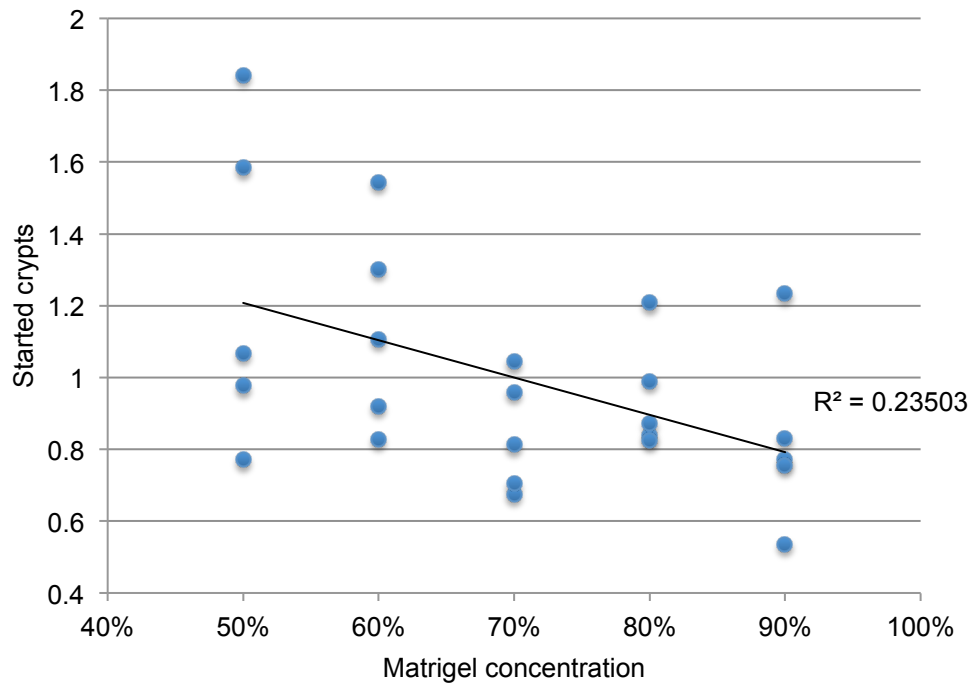


Figure 5. Normalized crypt starting efficiency in each Matrigel concentration (5 experimental rounds). The blue dots represent the results from each individual round. Trendline indicates the decrease of crypt growth when increasing the stiffness of ECM.

The Paneth cell numbers per single crypt were counted in order to see whether the stiffness also affected the Paneth cell differentiation. At the time of quantification, the crypts had been grown in culture for one week (approximately). Figure 6 presents the normalized data gathered altogether from five experimental rounds. There was a relatively wide range in the Paneth cell numbers between the rounds, however demonstrating that the stiffness might regulate the Paneth cell differentiation since their numbers show a decreasing trend with stiffer matrices.

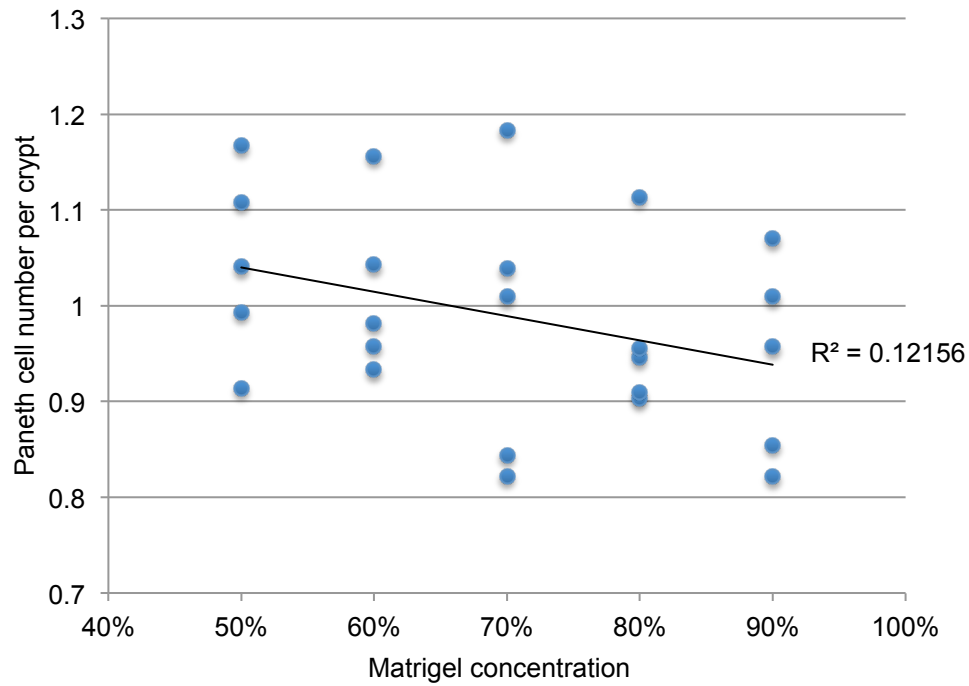


Figure 6. Normalized Paneth cell number per crypt in each Matrigel concentration (5 experimental rounds). The blue dots represent the results from each individual round. Trendline indicates a slight decrease in Paneth cell number when increasing the stiffness of ECM.

Simultaneously with the Paneth cell quantifications, the growing organoids were imaged in order to measure the crypt widths. The widths were measured from the bottom of the crypts (see Figure 4), from four experimental rounds. The results were normalized and are presented in Figure 7.

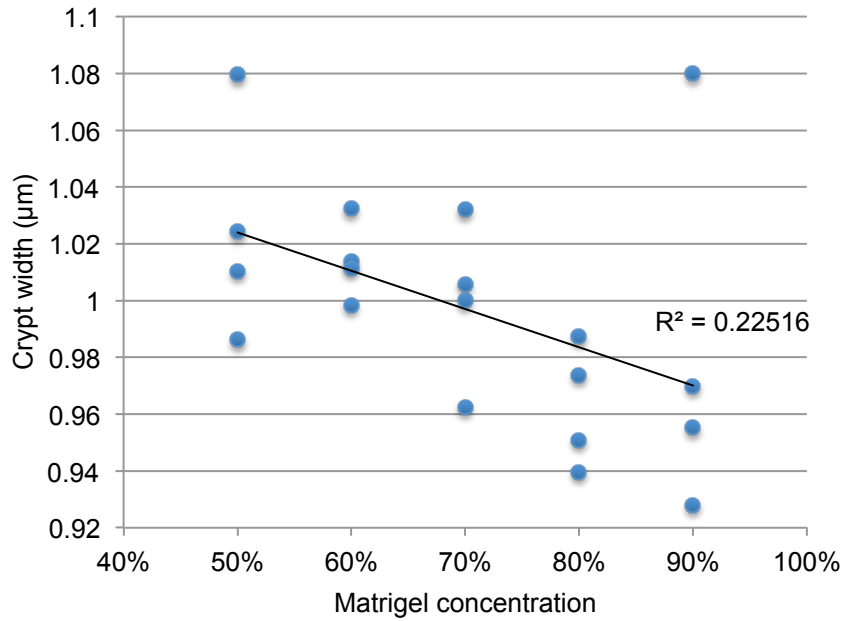


Figure 7. Average crypt width from each Matrigel concentration (4 experimental rounds). The blue dots represent the results from each individual round.

However, it was noticed that one data point was an outlier and when excluding it, there was a clear change in the results. Therefore, the width data is presented also without the outlier data point (Figure 8). Indeed, a marked correlation between crypt width and Matrigel concentration was observed when excluding the outlier data point ($p=0.03$, t-test result 50% vs. 90%).

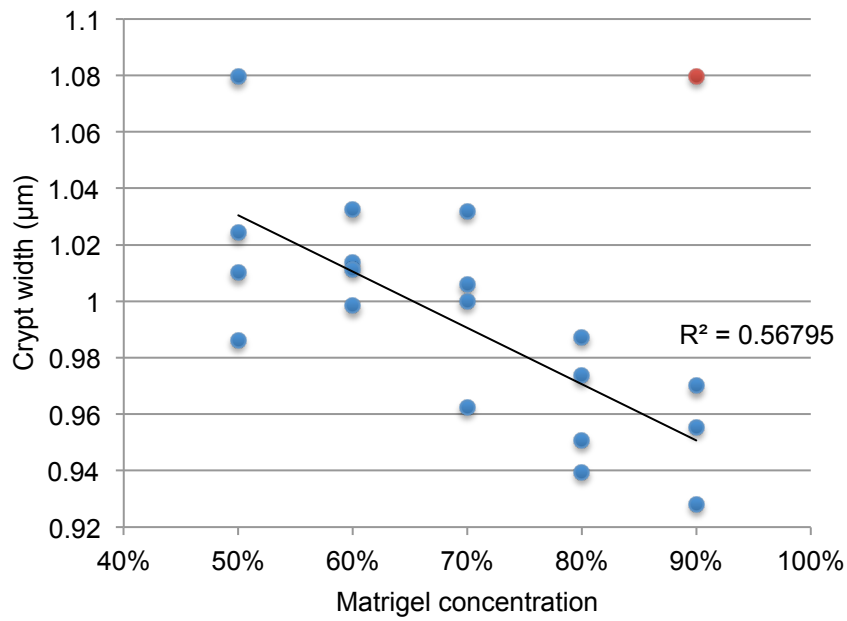


Figure 8. Average crypt width data excluding the outlier data point (represented in red).

In order to study the crypt formation in intestinal organoids, number of crypts in single organoids was quantified two days after subculturing. There was no remarkable difference in crypt formation between the culture concentrations, suggesting that the stiffness might not effect on crypt formation in observable level (Figure 9).

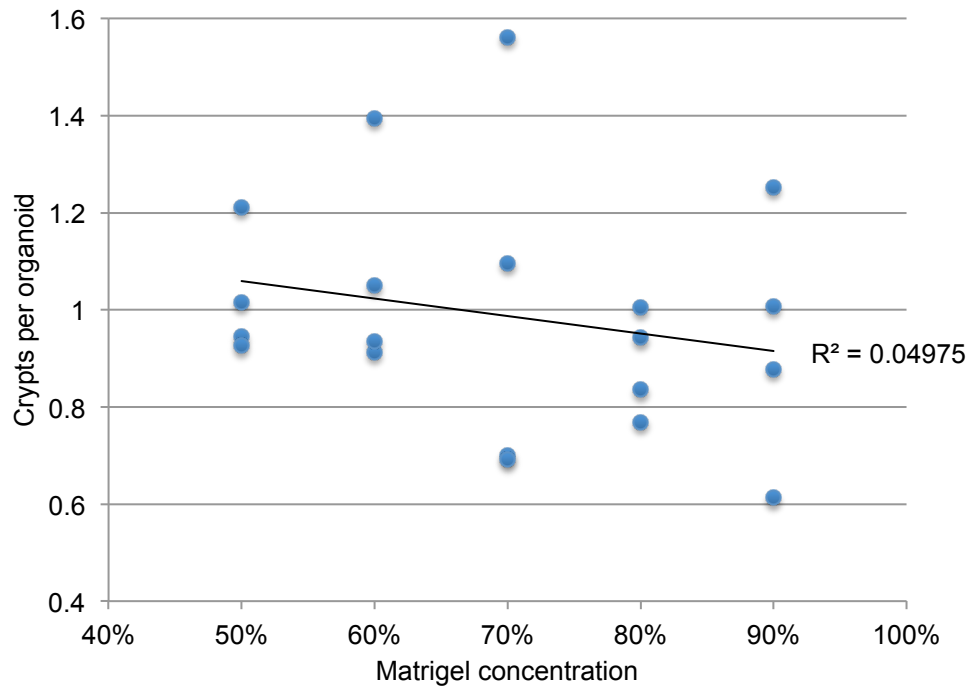


Figure 9. Data presenting the crypt amounts per single organoid. The blue dots represent the results from each individual round.

Due to their importance in ISC maintenance when cultured *in vitro*, the influence of the Paneth cells needed to be examined more closely. The Paneth cell numbers were compared to the data gathered from crypts in order to see, whether the Paneth cell number correlated the crypt width or the crypt formation in single organoid. The data used for these observations were not normalized; the numbers from each concentration on each round were only averaged. Even though the number of Paneth cells in single crypt and the number of crypts in single organoid both increase concurrently when the data are combined (Figure 10), the correlation cannot be declared on the basis of this study.

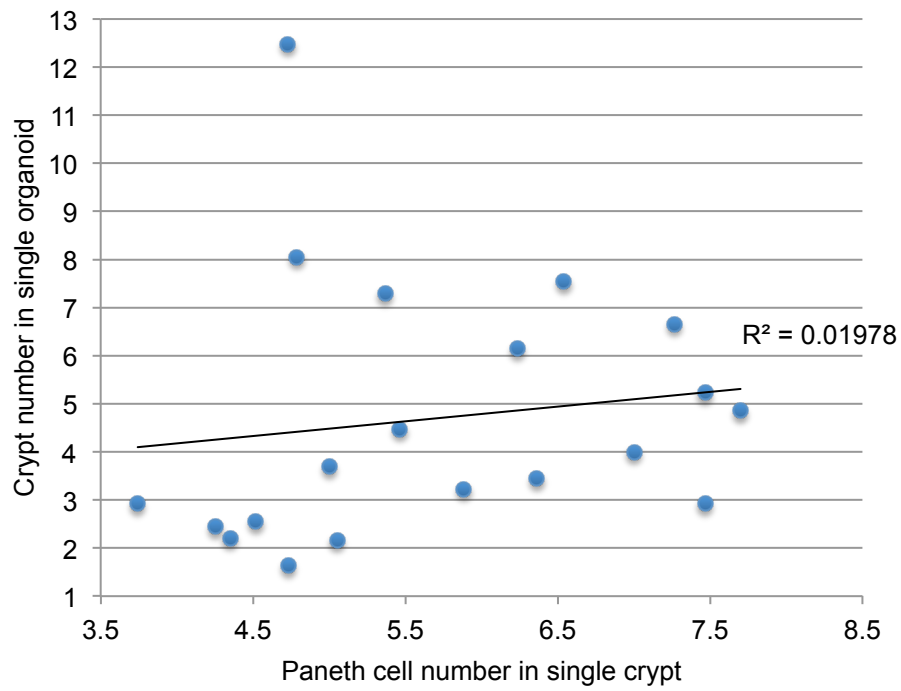


Figure 10. Paneth cell numbers combined with the crypt formation data.

Even though correlation between Paneth cell number and crypt width do clearly correlate (Figure 11), the data presented in Figure 10 do not allow conclusions regarding the impacts of Paneth cells on the crypt function.

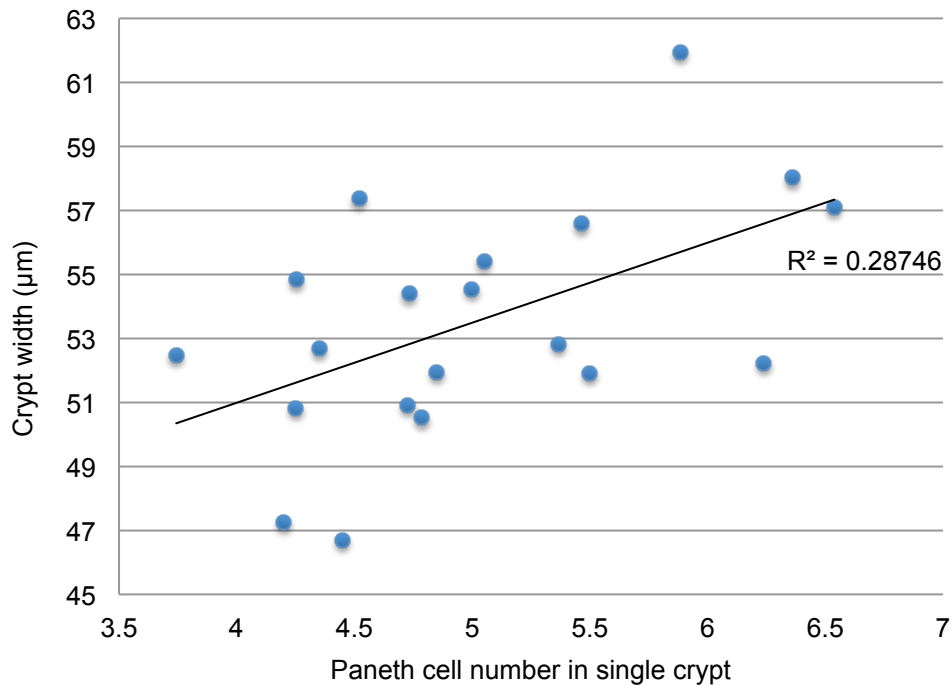


Figure 11. Paneth cell numbers combined with the crypt width data.

4.2 Cross-linking of Matrigel and modulation of matrix stiffness

After the experiments based on the Matrigel modifications, the aim was to increase the matrix stiffness by cross-linking Matrigel with calcium sulfate and alginate. The purpose was to create four different matrices where the laminin level remained stable while increasing stiffness of the matrix. Due to the difficulties and limitations in experiments, the crypts were unable to grow properly in these 3D cultures. Therefore, as no accurate and trustworthy data was acquired, these experiments are to be considered only as technical set-up experiments for this engineering thesis.

When mixing these components together with Matrigel, it was noticed that the calcium sulfate formed dark, crystal-like structures that filled the matrices and therefore, might have prevented the expected growth evidenced in the first experiments. Also, since the suspensions needed to be mixed sufficiently, the triturating caused remarkable amount of air bubbles inside the matrices and therefore the matrices did not attach to the bot-

tom of the wells but floated in the media. Moreover, both the air bubbles and the floating prevented proper counting, hence no reliable data was achieved.

The following figure is presented only for demonstrating the notable decrease in crypt growth and survival rate when culturing in a cross-linked matrices. The started crypts were counted on the next day after isolation and culture, gathering the results altogether from three experimental rounds. The results from each round were normalized in order to be able to compare the data (Figure 12). The right-most bar presents the control matrix without calcium sulfate and alginate (see Table 2), whereas the left-most bar presents the matrix without calcium sulfate but containing alginate (see Table 5). This data suggests that the isolated intestinal crypts started to grow and eventually form organoids more likely in loose matrix compared to stiffer cross-linked matrix.

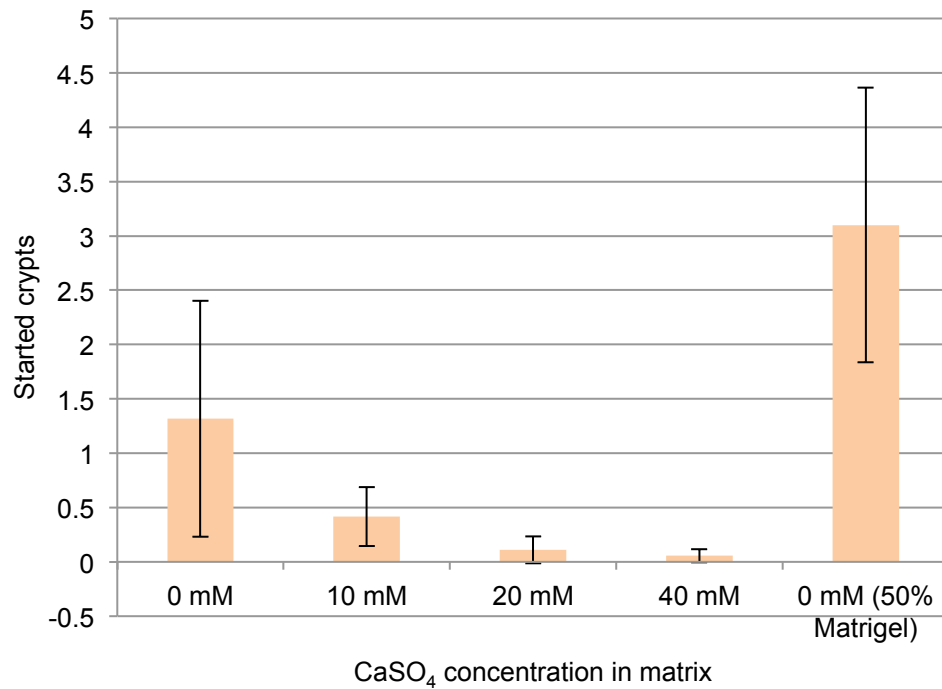


Figure 12. Normalized crypt starting efficiency in cross-linked matrices with varying CaSO₄ concentration (3 experimental rounds). The numbers indicate a rapid decrease in crypt formation when increasing CaSO₄ in matrix.

5 Discussion and conclusions

The purpose of this engineering thesis was to explore the relationship between the ECM stiffness and intestinal stem cell function. The experiments were carried out by culturing the isolated intestinal crypts in Matrigel-based 3D matrix that mimics laminin- and collagen-rich *in vivo* conditions. In order to study the stiffness precisely, the stiffness variations were created by both modifying the Matrigel-concentration and cross-linking the Matrigel with rigidity-increasing supplements.

As mentioned in the literature review, when cultured in 3D matrix, the ISCs grow into self-organizing intestinal organoids that resemble their original niche conditions. Nevertheless, the intestinal organoids can be generated only when the ISCs are in direct physical association with the Paneth cells. Indeed, the Paneth cells might play an important role in the control of ISC number, since it has been demonstrated that reducing the Paneth cell number decreases the number of stem cells. For that reason, the Paneth cell number was one of the key parameters examined in this thesis. When it comes to the ECM stiffness, there is a growing body of literature that recognizes the importance of ECM stiffness in terms of regulating the stem cell differentiation and the interaction between stem cells and the ECM. However, there has been no detailed investigation in terms of the ECM stiffness and ISC maintenance and this is the first time that Matrigel-based 3D matrices with various concentrations has been used to explore the ECM stiffness in regulation of ISC function. Therefore, there were no previous data to assimilate.

The first set of analyses examined the impact of the ECM stiffness to crypt survival and growth. As shown in Figure 5 and Figure 12, it seems that the isolated crypts survive and grow more likely in looser matrix, even though the observed difference between concentrations is not prominent. This is an interesting finding, demonstrating that substrate stiffness is likely a factor influencing renewal of the intestinal epithelium. With further investigation, this could be associated to existing knowledge of aging and tissue stiffening. As it can be seen from the data in Figure 6 and Figure 9, no clear decrease in average Paneth cell number in a single crypt, or crypt formation by single organoid was detected in these experiments. However, increasing ECM stiffness was interestingly observed to reduce to crypt width (see Figure 7). This was particularly clear, when the outlier data point was removed (see Figure 8), and the result is significant at the

$p=0.03$ level. The correlation between the crypt width and the concentration of Matrigel matrix is interesting, since the stiffness did not have effect the Paneth cell number per crypt as clearly (Figure 6). The decrease of crypt width might therefore at least partially result from changes in the shape of crypt cells. It is interesting to note, that such shape changes may possibly underlie the observed effects on crypt growth (Figure 5). However, further research with additional replicate samples is needed to explore more accurate results and functional data on a single cell type basis.

There are few sources of error worth mentioning in this thesis. Isolating and culturing primary cells is always slightly unstable, and yield of isolated crypts varied between the used mice. Since the protocols were unfamiliar, it took time to adopt adequate repetitions and suitable working methods in order to make isolating and subculturing proficient. Furthermore, the difficulties with the cross-linked matrix modulations prevented achieving trustworthy data.

Taken together, the objective whether the stiffness of the ECM has an influence on ISC function was achieved during this engineering thesis. As mentioned in the review of the literature, the ISCs are in close association with the Paneth cells *in vivo* and the existence of Paneth cells is required for growth and survival of ISCs in *in vitro* culture. Even though the numbers of ISC were not quantified precisely, the interaction between the matrix stiffness and renewal of intestinal epithelium was observed via other parameters. Since the matrix stiffness had an influence on Paneth cells and crypt formation, this engineering thesis substantiated the impact of ECM stiffness in ISC regulation.

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